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Title: DEVELOPMENT OF A METABOLOMIC APPROACH BASED ON URINE SAMPLES AND DIRECT INFUSION MASS SPECTROMETRY

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Abstract: The analysis of urine by direct infusion mass spectrometry suffers from ion suppression due to its high salt content and inter-sample variability caused by the differences in urine volume between persons. Thus, urine metabolomics requires a careful selection of the sample preparation procedure and a normalization strategy to deal with these problems. Several approaches were tested for metabolomic analysis of urine samples by direct infusion electrospray mass spectrometry, including solid phase extraction, liquid-liquid extraction and sample dilution. In addition, normalization of results based on conductivity values and statistical treatment was performed to minimize sample variability. Both urine dilution and solid phase extraction with mixed mode sorbent reduced considerably the salt content in urine providing comprehensive metabolomic fingerprints. Moreover, statistical data normalization enabled the correction of inter-samples physiological variability, improving the quality of results obtained. Therefore, high-throughput DI-ESI-MS fingerprinting of urine samples can be achieved with simple pretreatment procedures allowing the use of this non-invasive sampling in metabolomics. Finally, the optimized approach was tested in a pilot metabolomic investigation of urine samples from transgenic mice models of Alzheimer's disease (APP/PS1) in order to illustrate the potential of the methodology.

Dear Editor,

Please find enclosed new version of the manuscript “DEVELOPMENT OF A METABOLOMIC APPROACH BASED ON URINE SAMPLES AND DIRECT INFUSION MASS SPECTROMETRY” by R. Gonzalez-Dominguez, R. Castilla-Quintero, T. García-Barrera and J.L. Gómez-Ariza, to be published in Analytical Biochemistry.

The manuscript has been modified considering the comments and suggestions of referees and the new version has been rewritten marking the changes in yellow. The itemized response to referees is also included.

Yours sincerely

J.L. Gómez-Ariza

Dear Editor,

Please find enclosed the responses to referees remarks, which gave been considered and included in the new version of the manuscript.

Reviewer #1

The paper describes the characterization of a novel, but highly logical approach for the metabolic profiling of urine samples. Due to the need of high-throughput metabolic profiling techniques, the outlined MS-only approach is highly significant, since it enables the analysis of sample cohorts in the thousands to tens of thousands range. While the experimental plan and the description of methods is close to perfection, the authors neither present the data nor discuss its chemical/biological content. Hence, the paper cannot be considered for publication before the complete rewriting of the 'results and discussion' section. Since the data is already present, the required changes/additions are certainly doable within the revision timeframe of the Journal.

Specific comments:

1. Following LLE the extract is directly analysed by ESI-MS. To the best knowledge of the reviewer chloroform/methanol mixtures cannot be electrosprayed due to the mechanism of electrospray requiring conductive solvent. If these samples are introduced into a commercial ESI source featuring a metal spray capillary, one can certainly detect ions, however the ionization is associated with the corona discharge formed around the metal tip. For the sake of comparability, these samples should be subjected to solvent exchange and introduced in an appropriate solvent system.

Although it is advisable the use of polar conductive solvents to electrospray samples, the ionization is possible in presence of chloroform when salts and conductive solvents such as methanol are present in the mixture. A number of previous works reporting direct analysis of chloroform/methanol mixtures using electrospray mass spectrometry can be mentioned (PNAS, 1994, 91:10635; Anal Biochem, 2004, 330:317; RCMS, 2012, 26:835). In fact, we recently described a lipidomic approach for serum samples based on direct infusion mass spectrometry using chloroform extracts (J Pharm Biomed Anal, 2014, in press), where high ionization efficiency is achieved due to the presence of salts and conductive solvents in the mixture (methanol).

2. While electric conductance is an innovative basis for normalization, its correlation with the pH of urine must be discussed due to the presence of weak electrolytes.

The pH of urine samples was adjusted to 7 before sample treatment and analysis with two purposes:

1) Correction of inter-sample variability in conductivity values due to differential dissociation of electrolytes.
2) Improvement of reproducibility in SPE.

This point has been specified in the text.

3. Separation multivariate statistical space and predictive value of a model are highly important factors in metabolomics, however they cannot be used as the sole measures to compare metabolic profiling approaches. Presence and absence of a single component in the experimental and control groups, respectively, results in perfect separation and predictive values, but the goal of metabolomics is not to find single markers, but to study the entire metabolome. In this regard, the spectra must be interpreted, mass spectrometric peaks (at least a 30-50 representative ones) must be identified and the different sample preparation approaches have to be compared regarding their chemical/biochemical coverage of the metabolome. The professional reader is interested in the potential information content provided by the method among other performance parameters like sensitivity for certain constituents. Besides the lack of performance characterisation in the paper, one cannot properly evaluate the results without having information about the chemical content. In conclusion, the spectra need chemical interpretation, the statistical differences need to be presented in the light of chemical information and the various approaches need to be compared regarding these findings.

Metabolic fingerprints have been interpreted, and a new Table has been added containing the peak intensities of a number of representative metabolites using the different extraction procedures in order to evaluate the potential of each method for wide metabolome coverage.

4. The quality of the figures (even the hi-res ones) is extremely poor.

Quality of figures has been improved.

Reviewer #2

GENERAL COMMENTS

The manuscript describes an investigation of normalization procedures for untargeted metabolite profiling of single-spot urine samples using direct infusion-electrospray ionization-mass spectrometry (DI-ESI-MS). Although urine analyses provide a convenient biological fluid for clinical analyses, they are subject to significant biological variance due to different hydration status (diurnal cycle etc.) notably when 24 h urine collections are not performed. The authors compare different sample pretreatment (dilution and various extraction methods) and normalization procedures (conductivity, scaling) that maximize signal detection and enhance discrimination of gender/time of day human urine profiles with PLS-DA. In my opinion, there is no value of including the brief data from urine profiles of transgenic mouse models of Alzheimer's disease - ultimately it distracts form the main focus of the manuscript. Moreover, there is not interpretation of data or discussion of mass filtering/peak peaking algorithms used in DI-ESI-MS. Is only positive-ion mode detection used for metabolite fingerprinting; if so, why? In my opinion, the manuscript requires major revision to enhance its overall quality for the readership.

SPECIFIC COMMENTS:

1. Introduction: The authors are recommended to indicate that 24 h urine specimens are the "gold standard" for clinical analysis. Random single-spot urine collections are far more convenient yet subject to major biological variance. In addition to hydration status, other sources include diet, microflora, diurnal cycle etc. More emphasis on sources of variation are recommended in the introduction.

These corrections have been made in the Introduction section and also commented in the Conclusions.

2. Introduction: In addition to ion suppression (or enhancement!!) effects that complicate comparisons of different samples with variable matrix composition, the other major limitations of DI-ESI-MS include the lack of resolution of isobars/isomers and difficulty of quantification without stable-isotope internal standards. The latter constraints are improved when using complementary separation techniques with ESI-MS [Kuehnbaum et al. Chem Rev. 2013, 113: 2437-68].

A new sentence has been introduced to state these drawbacks associated with direct analysis instead of chromatographic separation prior to detection.

3. Introduction/Discussion: The authors need to provide a more in-depth discussion of how different normalization procedures (dilution VS conductivity-correction) are able to correct for ionization suppression or enhancement effects in urine. More importantly, recent studies have shown that "post-prandial" urine samples using standardized meals (breakfast) are an ideal way to reduce inter-subject variations for single-spot urine collections with DI-ESI-MS [Fave et al. Metabolomics, 2011, 7: 469-84]. The authors are strongly encouraged to highlight the limitations of their study design.

Normalization procedures were used to correct inter-sample biological variability. As stated in Introduction, conductivity is an indicator of total endogenous metabolic output and statistics can be used for scaling the spectra to the same virtual overall concentration, so they can be used to normalize biological variance due to physiological factors. On the other hand, the reduction of ion suppression-enhancement was addressed by using different sample treatment procedures to reduce the salt content (dilution, LLE, SPE).

Limitations of the study have been highlighted in the Conclusions section as well as a comment to Fave et al proposal.

4. Instrumentation/Data Analysis: The authors need to explain why DI-ESI-MS studies were not performed using negative ion mode given the large fraction of acidic/anionic metabolite sin urine. Also, there is insufficient detail regarding methods used for peak picking, isotope/in-source fragment filtering and blank correction due to the large fraction of artifact or spurious signals generated with DI-ESI-MS [Jankevics et al. Metabolomics 2012, 8: 29-36]. Also, what criteria were used to designate a molecular features of significance (precision, signal theresold (S/N), blank corrected etc.).

Analyses were also performed in negative mode, but due to the lower sensitivity (and consequently less number of metabolic features), discussion was only focused on data from positive ionization.

Parameters used for data pre-processing in the Markerview software have been included in the Materials and methods section.

5. Methodology/Results: It is not clear why the authors did not include a comparison of "creatinine-normalization" to ion signals (and PLS-DA) as this is the most common approach used for comparing single-spot urine samples used in literature and clinical practice. Without this gold-standard comparison, it is difficult to assess the value of other methods (conductivity etc.) used in this manuscript. Also, it would have been best to also include 24 h urine specimens from same subjects as standard reference from the two single-spot urine samples.
Creatinine normalization was discarded due to the growing evidence that creatinine excretion is not really constant, but depends on age, sex, diet, physical exercise, mental state or kidney impairment among other factors, as stated in the Introduction.
On the other hand, 24-h samples from subjects enrolled in the study were not considered given that metabolomic studies are usually performed on random collected samples, due to the easier sampling. Nevertheless, a comment about the limitation of do not use 24-h samples is included in the conclusions.

6. Methodology/Results: Can the authors normalize for ionic strength of urine matrix based on total ion current as an alternative to conductivity?
Normalization based on TIC was performed, and provided similar results to conductivity normalization.

7. Figures 1, 2: These figures do not provide much value in the manuscript and one could be retained (Fig. 3) as a comparison. However, the authors require additional supporting information in the form of a table that summarized all major peaks detected for transparency and data sharing while highlighting methods used for data processing of full-scan mass spectra.
Figures 1, 2 and 3 have been condensed into a single figure, and a new Table has been created containing major peaks detected in urine samples. More information is provided about data processing in Markerview software.

8. Figures 4 and 5: PLS-DA scores plots only provide evaluation of between-group discrimination. It is recommended that the authors discuss the variables (VIP ranking or loading plots) responsible for the increased discrimination with scaling or conductivity normalization. Although the emphasis of the manuscript is on metabolite fingerprinting, it would be insightful to identify key features.
A new figure has been added comparing VIP ranking plots form non-normalized data and statistically normalized data, which shows that the number of discriminant metabolites increases when data is normalized and demonstrates the potential of normalization to enhance group discrimination and subsequent identification of markers.

9. Figure 6/Discussion on transgenic mice: Since more rigorous examination of human urine samples are strongly recommended, the manuscript is not well served by including preliminary data on the transgenic mice.
Discussion on transgenic mice was provided in order to provide a more complete evaluation about the applicability of the normalization methods. However, we are open to modify it if necessary.

DEVELOPMENT OF A METABOLOMIC APPROACH BASED ON URINE SAMPLES AND DIRECT INFUSION MASS SPECTROMETRY

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Category: Mass spectrometry

Short title: Urine metabolomics by ESI-MS fingerprinting

Abstract

The analysis of urine by direct infusion mass spectrometry suffers from ion suppression due to its high salt content and inter-sample variability caused by the differences in urine volume between persons. Thus, urine metabolomics requires a careful selection of the sample preparation procedure and a normalization strategy to deal with these problems. Several approaches were tested for metabolomic analysis of urine samples by direct infusion electrospray mass spectrometry, including solid phase extraction, liquid-liquid extraction and sample dilution. In addition, normalization of results based on conductivity values and statistical treatment was performed to minimize sample variability. Both urine dilution and solid phase extraction with mixed mode sorbent reduced considerably the salt content in urine providing comprehensive metabolomic fingerprints. Moreover, statistical data normalization enabled the correction of inter-samples physiological variability, improving the quality of results obtained. Therefore, high-throughput DI-ESI-MS fingerprinting of urine samples can be achieved with simple pretreatment procedures allowing the use of this non-invasive sampling in metabolomics. Finally, the optimized approach was tested in a pilot metabolomic investigation of urine samples from transgenic mice models of Alzheimer's disease (APP/PS1) in order to illustrate the potential of the methodology.

Keywords: metabolomics, urine, direct infusion mass spectrometry, ion-suppression, normalization, APP/PS1 mice

Introduction

Metabolomics, defined as the comprehensive measurement of all metabolites in a sample, is a powerful tool in systems biology research, connecting the genome with the final phenotype via physiological development and through interactions with the environment [1]. The potential of this approach has been demonstrated in numerous fields including nutritional studies, disease biomarkers discovery and drug development [2,3]. Methodologically, the conventional use of nuclear magnetic resonance is being progressively replaced by mass spectrometry, since this technique offers several advantages such as higher sensitivity, capability for identification and quantification of metabolites, and suitability for application to complex samples [4]. The coupling of mass spectrometry with separation techniques is frequently reported in order to obtain simpler spectra and facilitate the interpretation of metabolic fingerprints [5], but these hyphenated approaches also present several drawbacks such as large time of analysis and analytical bias depending on the selected

metabolomic platform [6]. Alternatively, sample introduction by direct infusion into the mass spectrometry system (DIMS) has been recently proposed in disease studies with serum and plasma samples [7-11]. Although this approach presents several drawbacks related to the lack of resolution for isobars differentiation and difficulty of quantification without stable-isotope internal standards, the usefulness of this screening tool has been demonstrated in terms of wide metabolome coverage and fast analysis. For these reasons, the application of this high-throughput methodology to urine samples may be of great interest in disease diagnosis due to the easy availability and non-invasive sampling of this biological fluid, which reflects well the health state of organisms [12]. Urine samples are normally collected as 24-h samples in order to provide a complete picture of excretion, but random single-spot urine collection is more convenient in the clinical practice, despite the higher biological variance [13]. The analysis of urine samples by mass spectrometry presents two important problems related to ion suppression caused by this salty matrix [14] and inter-sample variability due to the differences in the volume of urine excreted between different individuals [15]. Therefore, sample preparation is critical in order to minimize ion suppression [16], removing salts from urine samples and minimizing possible perturbations of metabolites to obtain comprehensive fingerprints. Solid phase extraction (SPE) has become one of the most important techniques for the treatment of biological fluids in metabolomics [17], using both reverse and normal phases, and ion exchange matrixes [18,19]. However, the development of mixed mode sorbents, which involve multiple retention mechanisms by the incorporation of different ligands into the same sorbent, allows the extraction of a wider fraction of metabolites [20], and represents a promising alternative in sample treatment for metabolomic fingerprinting. In addition, liquid-liquid extraction (LLE) has also been considered for the treatment of this complex matrix for the separation of non-polar or low polar analytes, despite being a rare extraction technique in metabolomics [13]. On the other hand, normalization of results allows the reduction of biological variability caused by physiological factors such as hydration status, diet, microflora or diurnal cycle, for which is necessary the use of a correction parameter that does not systematically vary along the demographic groups of interest [21]. In this sense, measurements of ionic conductivity and osmolality have been proposed as indicators of total endogenous metabolic output, reflecting the dilution rate of urine samples [22]. Other conventional normalization parameter for urine is creatinine concentration since, under normal conditions, urinary creatinine excretion is relatively constant and measurable [23], although it varies depending on the age, sex, diet, physical exercise, mental state or kidney impairment among other factors [24]. In addition, different statistical methods for data preprocessing can also be performed to normalize results by scaling the spectra to the same virtual overall concentration [25,26].

There is few metabolomic studies related to the use of direct infusion mass spectrometry in urine samples, mainly in nutritional [27-29] and toxicological [30] areas, but there is still a need to delve into possible approaches that overcome the critical issues previously mentioned.

For this purpose in this work were evaluated several tools for sample treatment and data normalization in metabolomic analysis of urine samples using direct infusion electrospray mass spectrometry (DI-ESI-MS). It was studied several methods for urine treatment in order to reduce ion suppression, using solid phase extraction (SPE) with different sorbents, liquid-liquid extraction (LLE) and sample dilution. Moreover, ionic conductivity measurements and statistical preprocessing were compared for data normalization. In order to compare the effectiveness of these approaches for groups classification based on DIMS fingerprinting results, urine samples were studied from volunteers both men and women collected at different times of the day (fasting morning and after lunch). Finally, the most suitable approach was tested in a pilot study with urine samples from transgenic mice models of Alzheimer's disease (APP/PS1) in order to illustrate the potential of the methodology.

Materials and methods

Reagents and materials

All solvents used were HPLC-grade. Methanol and chloroform were purchased from Aldrich (Steinheim, Germany); acetic acid, ammonium acetate and dichloromethane were supplied by Merck (Darmstadt, Germany); and ammonia solution (32%) from VWR (Leuven, Belgium). Water was purified with a Milli-Q Gradient system (Millipore, Watford, UK). The SPE cartridges used were 3 ml columns, filled with 500 mg of DSC-18 (Supelco) for reverse phase extraction (C18), Bond Elut SI (Varian) for normal phase extraction (silica), and Isolute Multimode (IST) combining either non-polar (C18), strong cation exchange (SO_3^-) and strong anion exchange (NR_3^+) solid phases.

Sample collection

Two different sets of samples were used in this study. Human urine samples, collected from 11 volunteers (5 male and 6 female) fasting and after lunch, were used for evaluating the suitability of the different treatment and normalization procedures tested in this study. The second set was urine samples from transgenic mice models of Alzheimer's disease (APP/PS1) and wild-type controls, employed to validate the optimized methodology in a pilot study for the search of potential biomarkers of this disorder. APP/PS1 double transgenic mice (C57BL/6

background) were generated by crossing homozygotic PS1M146L transgenic mice with heterozygotic Thy1-APP751SL mice (Swedish: K670N, M671L and London: V717I FAD mutations) (Charles River, France), as previously reported [31]. On the other hand, age-matched wild-type mice of the same genetic background (C57BL/6) were purchased from Charles River Laboratory for their use as controls (WT). Male animals at 6 months of age were used for experiments (10 specimens per group). All samples were frozen immediately to -80ºC and stored until analysis.

Sample treatment

Three different strategies were tested for simultaneous desalting and extraction of metabolites from urine samples. In all cases, urine was thawed at room temperature and immediately centrifuged to remove particulate matter (6000 rpm, 10 minutes, 4 ºC). Before extraction, pH of these samples was adjusted to 7.

Sample dilution (SD). Urine was tenfold diluted with a 50 % (v/v) methanol/water mixture.

Liquid-liquid extraction (LLE). 500 µL of urine were mixed with the same volume of organic solvent (chloroform or dichloromethane), followed by vigorous vortexing for 5 min and centrifugation at 6000 rpm for 3 min at room temperature in order to collect the clean organic phase for analysis. Alternatively, 750 µL of a mixture of urine, methanol and organic solvent (1:1:1 v/v/v) was vigorously shaken by vortexing for 5 min and centrifuged at 6000 rpm for 3 min at room temperature.

Solid-phase extraction (SPE). Three SPE procedures were compared: reverse phase extraction (RP-SPE), normal phase extraction (NP-SPE) and mixed mode extraction (MM-SPE), this latter combining reverse phase sorbent with cationic and anionic exchangers. This extraction method requires sorbent wetting and equilibration before sample loading, followed by a washing step before the final elution of analytes. For this purpose, eluents were pumped through the column with a vacuum manifold (Visiprep^{TM}, Supelco). The solvents used for each SPE procedure are shown in Table 1.

Instrumentation

MS analyses were performed in a QSTAR XL Hybrid system (Applied Biosystems, Foster City, CA, USA) using an electrospray (ESI) source. The extracts were introduced into the mass spectrometer at 5 µL min^{-1} flow rates using an integrated apparatus pump and a 1000 µL volume Hamilton syringe. Data were obtained in positive ion mode, acquiring full scan spectra for 0.2 minutes in the m/z range 50-500 with 1.005 seconds of scan time. The ion spray voltage (IS) was set at 3300 V and nitrogen was used as curtain gas and nebulizer gas at flow rates about 1.13 L min^{-1} and 1.56 L min^{-1}, respectively. The source temperature was fixed at 60 ºC, with a declustering potential (DP) of 60 V and a focusing potential (FP) of 250 V. An AKTA-Prime system

(Amersham), integrated by a pump and conductivity detector, was used in flow injection mode for measurements of electrical conductivity. Extracts were injected in a 100 µl sample loop and were delivered to the detector by a carrier flow of water.

Data analysis

Markerview™ software (Applied Biosystems) was employed for peak picking and matching peaks across samples, in order to carry out the reduction of spectral data into a two-dimensional matrix of spectral peaks and peak intensities. For this, the peak search was done with a mass tolerance of 0.1 Da, and a minimum response of 10 counts was considered for filtering. In addition, missing values were substituted with the mean of the non-missing values across all samples for that peak. Then, data was processed in SIMCA-P™ software (version 11.5, published by UMetrics AB, Umeå, Sweden). Partial least squares discriminant analysis (PLS-DA) was performed to check the potential of the metabolomic approaches applied to urine samples for classification of samples in the groups of study. In addition, quality of the models was assessed by the R^2 and Q^2 values, supplied by the software, which provide information about the class separation and predictive power of the model, respectively. These parameters are ranged between 0 and 1, and they indicate the variance explained by the model for all the data analyzed (R^2) and this variance in a test set by cross-validation (Q^2). Statistical data normalization was carried out in the R software (freeware, http://www.r-project.org), using the locally weighted scatter plot smoothing (LOESS) normalization method, which adjusts the local median of log fold changes of peak intensities between samples in a data set to be approximately zero across the whole peak intensity range [32]. In addition, the heteroscedasticity of noise in peak intensities, characterized by increasing technical variance as a function of increased signal intensity, was corrected by means of a logarithmic-based variance-stabilization transformation, to make peak intensity consistent across the whole intensity range [32,33].

Results and discussion

The comparison of metabolomic profiles obtained with the different tested procedures demonstrated the importance of a suitable choice of the extraction protocol for obtaining comprehensive results when DIMS is used as metabolomic approach. In addition, a considerable inter-sample variability was observed, showing the need of using normalization strategies. In order to determine the most suitable approach for urine metabolomics, statistical analysis (PLS-DA) of data from the first sample set (men and women, and different times of collection - morning and midday) was performed for sample classification according to the groups of study.

Then, the applicability of the optimized methodology was checked in a pilot study with APP/PS1 transgenic mice and wild-type controls.

Classification of human urine samples using non-normalized results

Application of treatment procedures previously described to urine samples provided different DIMS metabolomic profiles, showing that extraction of metabolites is strongly dependent on the selectivity of technique, which may result in biased fingerprinting. Thus, data obtained by simple dilution of urine samples with 50% water/methanol mixture could be considered as reference fingerprint of this type of sample, since no losses of metabolites occurs with this treatment, which allows obtaining comprehensive spectral profiles (Fig. 1a). The optimal dilution factor was estimated considering the number of peaks and total intensity of signal in overall mass spectrum resulting after successive dilutions. In this way, only a dilution higher than tenfold provoked a significant decrease in sensitivity of metabolomic profiles, so this value was selected for further experiences. When urine was liquid-liquid extracted with chloroform or dichloromethane very similar metabolic profiles were obtained, but the number and intensities of signals increase when the extraction was performed adding methanol (Fig. 1b, for chloroform-methanol), since methanol acts as "phase transfer agent" promoting the extraction of more polar analytes from aqueous to organic phase. However, comparing these results with those from urine dilution (Fig. 1a), the number of extracted metabolites considerably decreased due to the loss of polar compounds in these non-aqueous solvents, such as chloroform or dichloromethane. Finally, when urine is solid-phase extracted, different profiles are obtained depending on the use of normal or reverse extraction phases, since the extraction is somewhat selective towards polar or lipophilic metabolites respectively, as is shown in Fig. 1c and 1d. On the other hand, when the mixed mode sorbent is used, the mass spectra of the extract exhibits a higher number of signals (Fig. 1e), similarly to that obtained from diluted samples, demonstrating the capability of the mixed mode sorbent for the global recovery of urinary metabolites.

In addition to the visual inspection of metabolomic profiles obtained for each pretreatment procedure, multivariate statistics allowed evaluating the suitability of the different urine extraction protocols for metabolomic fingerprinting. In this way, MS profiles were submitted to partial least squares discriminant analysis, and 3D scores plots of the three first components (in five components models) were represented in order to visualize trending and grouping of samples (Fig. 2, for the best procedures in terms of metabolite coverage observed in MS profiles). In this way, the best discrimination between the four groups was obtained with sample dilution and mixed mode SPE extraction (Fig. 2a and 2c). Moreover, the higher statistical quality of these models can be corroborated by the parameters of separation and predictive power (shown in Figure 4,

under the respective scores plots), which also support the advantage of SD and MM-SPE over other procedures. Furthermore, in order to evaluate the chemical coverage of the metabolome obtained with these procedures compared by multivariate statistics (SD, LLE, MM-SPE), a number of representative metabolites from metabolomic fingerprints were identified (Table 2). As can be observed, all these metabolites were detected using the three extraction procedures, but higher sensitivity was obtained with diluted samples and, to a lesser extent, solid phase extraction (in terms of peak intensity), corroborating the higher potential of these methods for high-throughput metabolomic analysis.

Classification of human urine samples after ionic conductivity normalization

The ionic conductivity of urine extracts is a parameter of great interest, since provides information about effectiveness of salts removing from the sample after treatment, and allows data normalization in relation to physiological dilution rate of urine. Thus, conductivity was measured in diluted samples and extracts from SPE, resulting in high dispersion of values for urine dilution samples (SD), that range from 0.08 to 0.56 mS cm^{-1} with RSD of 39 %, which denotes a great physiological variability between samples, and low conductivity in reverse and normal phase extracts (RP-SPE and NP-SPE), in which the mean values of conductivity are 0.20 and 0.08 mS cm^{-1}, although in this case the conductivity is difficult to normalize due to the wide variability of results (RSD of 48 and 63% for RP-SPE and NP-SPE, respectively). In mixed mode extraction (MM-SPE), the conductivity, with mean values of 0.49 mS cm^{-1}, is difficult to reduce probably due to the use of buffer solutions in the elution step, however, the reproducibility is better (RSD=18%).

Therefore, it can be concluded that urine desalting is more effective by using reverse and normal phase extraction, but the reproducibility is considerably higher with mixed mode sorbents. Then, these values of ionic conductivity were employed for the normalization of signal intensities in mass spectra to improve the discrimination between the groups of study. However, for diluted samples and the extracts from RP-SPE and NP-SPE treatments, the separation between groups was reduced comparing with non-normalized data, while for MM-SPE the statistical model obtained after normalization was analogous to that non-normalized, with R^2=0.979 and Q^2=0.455 (Fig. 3a and 3b).

Classification of human urine samples after statistical normalization

Finally, statistical normalization of data by LOESS method was used in order to obtain optimal scaling factors for each sample based on the complete dataset, assuming that metabolomic profiles should be similar between different samples in absence of differences in the excreted volume of urine. This approach provided slightly

better discrimination models compared to non-normalized ones, with higher R^2 and Q^2 values and scores plots with better separation between groups (Fig. 3c, 3d and 3e). Furthermore, discrimination was clearer in models built with data from MM-SPE (R^2=0.988, Q^2=0.517) and diluted samples (SD) (R^2=0.983, Q^2=0.559), similarly to that found with non-standardized data.

In summary, it could be concluded that sample dilution is the most suitable procedure for urine treatment before analysis by direct infusion mass spectrometry, in terms of simplicity, sensitivity and metabolomic coverage (Table 2). Furthermore, statistical normalization by LOESS showed the higher potential for correcting inter-sample variability, facilitating group discrimination in PLS-DA models. Thereby, it can be observed that the number of discriminant metabolites (VIP>2.0) increases from 18 to 25 when VIP ranking plots from non-normalized data and results statistically normalized are compared (Fig. 4), demonstrating the usefulness of LOESS normalization to extract the relevant biological information from this data set.

Application of DIMS metabolomics to a pilot study with Alzheimer's disease transgenic mice

Direct analysis of diluted samples followed by statistical normalization of data (LOESS) has been demonstrated to be the most reliable and simple approach for high throughput fingerprinting of urine by direct infusion electrospray mass spectrometry, considering results shown in previous sections. In order to evaluate the potential of this methodology in a real metabolomic investigation, urine samples from transgenic mice of Alzheimer's disease (APP/PS1) and wild-type controls were analyzed in the same way. Alzheimer's disease (AD) is the most common neurodegenerative disorder worldwide, whose exact etiology and pathogenesis are not clear. For a better understanding of pathogenic mechanisms occurring, transgenic mouse models have been developed, among which the double mutant transgenic line APP/PS1 has gained great importance [34]. The potential of metabolomics for the discovery of biomarkers in AD has been previously reported [35], but the use of urine has been only scarcely considered [36]. Thus, the application of the methodology optimized in the present work could represent a new tool in the search of potential biomarkers using a very simple and easily available sample in clinical laboratory as is urine. In this pilot study, ten specimens were included per group (transgenic mice - TG; and wild-type controls - WT). Partial least squares discriminant analysis provided a good classification of samples in the two expected groups (Fig. 5a), with very high statistic parameters of class separation and predictive power (R^2=0.999 and Q^2=0.99). In addition, numerous signals varied significantly between groups, as shown in the Variable Importance plot (Fig. 5b). Therefore, these preliminary results demonstrate the potential of high throughput direct infusion metabolomics in urine samples for the study of complex diseases such as AD, and open the door to future studies for biomarkers discovery based in this fluid.

Conclusions

The importance of sample treatment and data normalization in the analysis of urine by direct infusion mass spectrometry for metabolomic studies has been evaluated. Ion suppression must be reduced in order to obtain valuable results, for which different protocols of sample preparation were studied. Simple tenfold dilution of samples and solid phase extraction in a mixed-mode sorbent (MM-SPE) provided the best results for comprehensive metabolomic fingerprinting and grouping of samples in the subsequent statistical analysis. On the other hand, liquid-liquid extraction with organic solvents and solid phase extraction with reverse and normal phases, which involves a more selective recovery of metabolites, provided more biased metabolomic profiles. Moreover, the suitability of a normalization step was evaluated in order to correct the physiological variability of urine samples. Statistical processing of non-normalized data allowed discrimination between groups in diluted urine and MM-SPE extracts. Normalization based on conductivity provided good statistical models only for MM-SPE extracts, in which conductivity measures showed that this is the only procedure with reproducible desalting power. On the other hand, statistical normalization by LOESS showed the higher potential for these purposes, improving significantly the quality of the metabolomic data. However, a limitation of this study is the absence of a protocol for diet standardization prior to sample collection, as proposed by Favé et al. in order to reduce inter-subject variability [37]. Furthermore, 24-h urine samples from the same subjects were not available in our study design in order to compare with samples randomly collected, although these latter are usually more employed in metabolomic studies due to easier sampling. Finally, the optimized approach based on direct analysis of diluted samples followed by statistical normalization of data was applied to a pilot study with samples from APP/PS1 transgenic mice, demonstrating the potential of this methodology for high throughput metabolomics of urine samples.

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References

[1] O. Fiehn, Metabolomics–the link between genotypes and phenotypes, Plant Mol. Biol. 48 (2002) 155-171.

[2] D.S. Wishart, Metabolomics: applications to food science and nutrition research, Trends Food Sci. Tech. 19 (2008) 482-493.

[3] P. Goldsmith, H. Fenton, G. Morris-Stiff, N. Ahmad, J. Fisher, K.R. Prasad, Metabonomics: A useful tool for the future surgeon, J. Surg. Res. 160 (2010) 122-132.

[4] K. Dettmer, P.A. Aronov, B.D. Hammock, Mass spectrometry-based metabolomics, Mass Spectrom. Rev. 26 (2007) 51-78.

[5] H.J. Issaq, E. Abbott, T.D. Veenstra, Utility of separation science in metabolomic studies, J. Sep. Sci. 31 (2008) 1936-1947.

[6] W.B. Dunn, Current trends and future requirements for the mass spectrometric investigation of microbial, mammalian and plant metabolomes, Phys. Biol. 5 (2008) 011001.

[7] L. Lin, Q. Yu, X. Yan, W. Hang, J. Zheng, J. Xing, B. Huang, Direct infusion mass spectrometry or liquid chromatography mass spectrometry for human metabonomics? A serum metabonomic study of kidney cancer, Analyst 135 (2010) 2970-2978.

[8] P.G. Lokhov, M.I. Dashtiev, S.A. Moshkovskii, A.I. Archakov, Metabolite profiling of blood plasma of patients with prostate cancer, Metabolomics 6 (2010) 156-163.

[9] P.G. Lokhov, O.N. Kharybin, A.I. Archakov, Diagnosis of lung cancer based on direct-infusion electrospray mass spectrometry of blood plasma metabolites, Int. J. Mass Spectrom. 309 (2012) 200-205.

[10] R. Gonzalez-Dominguez, T. Garcia-Barrera, J.L. Gomez-Ariza, Metabolomic approach to Alzheimer's disease diagnosis based on mass spectrometry, Chem. Papers 66 (2012) 829–835.

[11] R. Gonzalez-Dominguez, T. Garcia-Barrera, J.L. Gomez-Ariza, Combination of metabolomic and phospholipid-profiling approaches for the study of Alzheimer's disease, J. Proteomics (2014) *in press.*

[12] J.A. Simerville, W.C Maxted, J.J. Pahira, Urinalysis: A comprehensive review, Am. Fam. Physician 71 (2005) 1153-1162.

[13] M.A. Fernández-Peralbo, M.D. Luque de Castro, Preparation of urine samples prior to targeted or untargeted metabolomics mass-spectrometry analysis, TrAC-Trends Anal. Chem. 41 (2012) 75-85.

[14] T.M. Annesley, Ion suppression in mass spectrometry, Clin. Chem. 49 (2003) 1041-1044.

[15] S.S. Que-Hee, Biological monitoring: An introduction, John Wiley & Sons, New York, 1993.

[16] R. Bonfiglio, R.C. King, T.V. Olah, K. Merkle, The effects of sample preparation methods on the variability of the electrospray ionization response for model drug compounds, Rapid Commun. Mass Spectrom. 13 (1999) 1175-1185.

[17] V. Walker, G.A. Mills, Solid-phase extraction in clinical biochemistry, Annals Clin. Biochem. 39 (2002) 464-477.

[18] F. Michopoulos, L. Lai, H. Gika, G. Theodoridis, I. Wilson, UPLC–MS-based analysis of human plasma for metabonomics using solvent precipitation or solid phase extraction, J. Proteome Res. 8 (2009) 2114-2121.

[19] H. Idborg-Bjorkman, P. Edlund, O.M. Kvalheim, I. Schuppe-Koistinen, S.P. Jacobsson, Screening of biomarkers in rat urine using LC/electrospray ionization-MS and two-way data analysis, Anal. Chem. 75 (2003) 4784-4792.

[20] A. Namera, S. Yamamoto, T. Saito, S. Miyazaki, H. Oikawa, A. Nakamoto, M. Nagao, Simultaneous extraction of acidic and basic drugs from urine using mixed-mode monolithic silica spin column bonded with octadecyl and cation-exchange group, J. Sep. Sci. 34 (2011) 2232-2239.

[21] B.M. Warrack, S. Hnatyshyna, K.H. Otta, M.D. Reilya, M. Sanders, H. Zhang, D.M. Drexler, Normalization strategies for metabonomic analysis of urine samples, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 877 (2009) 547-552.

[22] V. Chadha, U. Garg, U.S. Alon, Measurement of urinary concentration: a critical appraisal of methodologies, Pediatr. Nephrol. 16 (2001) 374-382.

[23] E.J. Saude, D. Adamko, B.H. Rowe, T. Marrie, B.D. Sykes, Variation of metabolites in normal human urine, Metabolomics 3 (2007) 439-451.

[24] D. Ryan, K. Robards, P.D. Prenzler, M. Kendall, Recent and potential developments in the analysis of urine: A review, Anal. Chim. Acta. 684 (2011) 17-29.

[25] F. Dieterle, A. Ross, G. Schlotterbeck, H. Senn, Probabilistic quotient normalization as robust method to account for dilution of complex biological mixtures. Aplication in ^{1}H-NMR metabolomics, Anal. Chem. 78 (2006) 4281-4290.

[26] S.M. Kohl, M.S. Klein, J. Hochrein, P.J. Oefner, R. Spang, W. Gronwald, State-of-the art data normalization methods improve NMR-based metabolomic analysis, Metabolomics 8 (2012) 146-160.

[27] M. Beckmann, D.P. Enot, D.P. Overy, I.M. Scott, P.G. Jones, D. Allaway, J. Draper, Metabolite fingerprinting of urine suggests breed-specific dietary metabolism differences in domestic dogs, Br. J. Nutr. 103 (2010) 1127-1138.

[28] A.J. Lloyd, G. Favé, M. Beckmann, W. Lin, K. Tailliart, L. Xie, J.C. Mathers, J. Draper, Use of mass spectrometry fingerprinting to identify urinary metabolites after consumption of specific foods, Am. J. Clin. Nutr. 94 (2011) 981-991.

[29] G. Favé, M. Beckmann, A.J. Lloyd, S. Zhou, G. Harold, W. Lin, K. Tailliart, L. Xie, J. Draper, J.C. Mathers, Development and validation of a standardized protocol to monitor human dietary exposure by metabolite fingerprinting of urine samples, Metabolomics 7 (2011) 469-484.

[30] M. Hasegawa, S. Takenaka, M. Kuwamura, J. Yamate, S. Tsuyama, Urinary metabolic fingerprinting for amiodarone-induced phospholipidosis in rats using FT-ICR MS, Exp. Toxicol. Pathol. 59 (2007) 115-120.

[31] V. Blanchard, S. Moussaoui, C. Czech, N. Touchet, B. Bonici, M. Planche, T. Canton, I. Jedidi, M. Gohin, O. Wirths, T.A. Bayer, D. Langui, C. Duyckaerts, G. Tremp, L. Pradier, Time sequence of maturation of dystrophic neuritis associated with Abeta deposits in APP/PS1 transgenic mice, Exp. Neurol. 184 (2003) 247-263.

[32] K.A. Veselkov, L.K. Vingara, P. Masson, S.L. Robinette, E. Want, J.V. Li, R.H. Barton, C. Boursier-Neyret, B. Walther, T.M. Ebbels, I. Pelczer, E. Holmes, J.C. Lindon, J.K. Nicholson, Optimized preprocessing of ultra-performance liquid chromatography/mass spectrometry urinary metabolic profiles for improved information recovery, Anal. Chem. 83 (2011) 5864-5872.

[33] M. Sysi-Aho, M. Katajamaa, L. Yetukuri, M. Orešič, Normalization method for metabolomics data using optimal selection of multiple internal standards, BMC Bioinformatics 8 (2007) 93-110.

[34] A.M. Hall, E.D. Roberson, Mouse models of Alzheimer's disease, Brain Res. Bull. 88 (2012) 3-12.

[35] C. Ibáñez, C. Simó, A. Cifuentes, Metabolomics in Alzheimer's disease research, Electrophoresis 34 (2013) 2799-2811.

[36] K. Fukuhara, A. Ohno, Y. Ota, Y. Senoo, K. Maekawa, H. Okuda, M. Kurihara, A. Okuno, S. Niida, Y. Saito, O. Takikawa, NMR-based metabolomics of urine in a mouse model of Alzheimer's disease: identification of oxidative stress biomarkers, J. Clin. Biochem. Nutr. 52 (2013) 133-138.

[37] G. Favé, M. Beckmann, A.J. Lloyd, S. Zhou, G. Harold, W. Lin, K. Tailliart, L. Xie, J. Draper, J.C. Mathers, Development and validation of a standardized protocol to monitor human dietary exposure by metabolite fingerprinting of urine samples, Metabolomics 7 (2011) 469-484.

Figure legends

Fig. 1 Metabolomic fingerprints from diluted urine (a), LLE extract using chloroform/methanol (b) and SPE extracts using NP-SPE (c), RP-SPE (d) and MM-SPE (e)

Fig. 2 3D scores plots and statistical parameters of non-normalized data for: diluted samples (a), LLE using chloroform/methanol (b), and MM-SPE (c)

Fig. 3 3D scores plots of normalized data based on ionic conductivity for: diluted samples (a), and MM-SPE (b); and statistically normalized for: diluted samples (c), LLE using chloroform/methanol (d), and MM-SPE (e)

Fig. 4 Variable Importance plots for non-normalized data (a) and data normalized with LOESS (b)

Fig. 5 Partial least squares discriminant analysis of data from the pilot study with Alzheimer's disease transgenic mice: scores plot (a), and Variable Importance plot (b)

Table 1.- Experimental condition for SPE procedures

	RP-SPE	**NP-SPE**	**MM-SPE**
Conditioning	2ml methanol	2ml methanol	2ml methanol
Equilibration	2ml water	2ml methanol 5%	-
Sample loading	1ml urine	1ml urine	1.5ml urine
Washing	2ml water	2ml methanol 95%	2ml water
Elution	1ml methanol	1ml methanol 5%	1. 0.5ml methanol 2. 0.5ml ammonium acetate 10 mM (pH 3) 3. 0.5ml ammonia 5% (in methanol)

Table 2 Peak intensities of representative metabolites identified in urine samples detected by sample dilution (SD), liquid-liquid extraction (LLE) and mixed mode solid-phase extraction (MM-SPE)

metabolite	m/z (Da)	peak intensity		
		SD	LLE	MM-SPE
trimethylamine	60.079	278.7	1.5	54.4
urea	61.037	13361.6	60.8	380.4
trimethylamine N-oxide	76.074	5813.9	92.8	686.1
alanine	90.055	121.6	0.3	7.0
glycerol	93.051	26.0	0.6	11.6
choline	104.105	187.7	3.3	53.9
serine	106.054	39.6	11.8	49.0
creatinine	114.064	18741.6	2137.2	15650.5
proline	116.075	133.5	14.5	77.5
valine	118.082	224.7	5.8	65.5
threonine	120.069	29.9	4.2	36.4
cysteine	122.031	55.9	5.3	30.9
taurine	126.025	128.5	16.0	55.7
pipecolic acid	130.098	229.9	8.7	71.1
creatine	132.074	440.0	7.2	72.4
aspartic acid	134.051	170.3	2.5	24.9
trigonelline	138.052	1804.4	8.9	233.6
histidine	156.074	1262.1	6.0	87.8
carnitine	162.108	1458.9	2.9	152.4
phenylalanine	166.082	5391.8	20.4	472.2
methylhistidine	170.086	1840.4	8.1	113.9
arginine	175.116	426.8	2.7	42.4
N-acetyl-aspartic acid	176.055	272.1	4.3	20.2

glucose	181.079	527.4	38.0	811.2
tyrosine	182.076	1985.7	11.4	330.8
phosphocholine	184.081	378.2	43.6	133.9
acetylcarnitine	204.118	532.1	4.2	99.7
carnosine	227.122	1183.3	86.5	459.1
uridine	245.081	316.3	4.1	44.1
uridine monophosphate	325.049	492.4	0.5	196.2

DEVELOPMENT OF A METABOLOMIC APPROACH BASED ON URINE SAMPLES AND DIRECT INFUSION MASS SPECTROMETRY

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Category: Mass spectrometry

Short title: Urine metabolomics by ESI-MS fingerprinting

Abstract

The analysis of urine by direct infusion mass spectrometry suffers from ion suppression due to its high salt content and inter-sample variability caused by the differences in urine volume between persons. Thus, urine metabolomics requires a careful selection of the sample preparation procedure and a normalization strategy to deal with these problems. Several approaches were tested for metabolomic analysis of urine samples by direct infusion electrospray mass spectrometry, including solid phase extraction, liquid-liquid extraction and sample dilution. In addition, normalization of results based on conductivity values and statistical treatment was performed to minimize sample variability. Both urine dilution and solid phase extraction with mixed mode sorbent reduced considerably the salt content in urine providing comprehensive metabolomic fingerprints. Moreover, statistical data normalization enabled the correction of inter-samples physiological variability, improving the quality of results obtained. Therefore, high-throughput DI-ESI-MS fingerprinting of urine samples can be achieved with simple pretreatment procedures allowing the use of this non-invasive sampling in metabolomics. Finally, the optimized approach was tested in a pilot metabolomic investigation of urine samples from transgenic mice models of Alzheimer's disease (APP/PS1) in order to illustrate the potential of the methodology.

Keywords: metabolomics, urine, direct infusion mass spectrometry, ion-suppression, normalization, APP/PS1 mice

Introduction

Metabolomics, defined as the comprehensive measurement of all metabolites in a sample, is a powerful tool in systems biology research, connecting the genome with the final phenotype via physiological development and through interactions with the environment [1]. The potential of this approach has been demonstrated in numerous fields including nutritional studies, disease biomarkers discovery and drug development [2,3]. Methodologically, the conventional use of nuclear magnetic resonance is being progressively replaced by mass spectrometry, since this technique offers several advantages such as higher sensitivity, capability for identification and quantification of metabolites, and suitability for application to complex samples [4]. The coupling of mass spectrometry with separation techniques is frequently reported in order to obtain simpler spectra and facilitate the interpretation of metabolic fingerprints [5], but these hyphenated approaches also present several drawbacks such as large time of analysis and analytical bias depending on the selected

metabolomic platform [6]. Alternatively, sample introduction by direct infusion into the mass spectrometry system (DIMS) has been recently proposed in disease studies with serum and plasma samples [7-11]. Although this approach presents several drawbacks related to the lack of resolution for isobars differentiation and difficulty of quantification without stable-isotope internal standards, the usefulness of this screening tool has been demonstrated in terms of wide metabolome coverage and fast analysis. For these reasons, the application of this high-throughput methodology to urine samples may be of great interest in disease diagnosis due to the easy availability and non-invasive sampling of this biological fluid, which reflects well the health state of organisms [12]. Urine samples are normally collected as 24-h samples in order to provide a complete picture of excretion, but random single-spot urine collection is more convenient in the clinical practice, despite the higher biological variance [13]. The analysis of urine samples by mass spectrometry presents two important problems related to ion suppression caused by this salty matrix [14] and inter-sample variability due to the differences in the volume of urine excreted between different individuals [15]. Therefore, sample preparation is critical in order to minimize ion suppression [16], removing salts from urine samples and minimizing possible perturbations of metabolites to obtain comprehensive fingerprints. Solid phase extraction (SPE) has become one of the most important techniques for the treatment of biological fluids in metabolomics [17], using both reverse and normal phases, and ion exchange matrixes [18,19]. However, the development of mixed mode sorbents, which involve multiple retention mechanisms by the incorporation of different ligands into the same sorbent, allows the extraction of a wider fraction of metabolites [20], and represents a promising alternative in sample treatment for metabolomic fingerprinting. In addition, liquid-liquid extraction (LLE) has also been considered for the treatment of this complex matrix for the separation of non-polar or low polar analytes, despite being a rare extraction technique in metabolomics [13]. On the other hand, normalization of results allows the reduction of biological variability caused by physiological factors such as hydration status, diet, microflora or diurnal cycle, for which is necessary the use of a correction parameter that does not systematically vary along the demographic groups of interest [21]. In this sense, measurements of ionic conductivity and osmolality have been proposed as indicators of total endogenous metabolic output, reflecting the dilution rate of urine samples [22]. Other conventional normalization parameter for urine is creatinine concentration since, under normal conditions, urinary creatinine excretion is relatively constant and measurable [23], although it varies depending on the age, sex, diet, physical exercise, mental state or kidney impairment among other factors [24]. In addition, different statistical methods for data preprocessing can also be performed to normalize results by scaling the spectra to the same virtual overall concentration [25,26].

There is few metabolomic studies related to the use of direct infusion mass spectrometry in urine samples, mainly in nutritional [27-29] and toxicological [30] areas, but there is still a need to delve into possible approaches that overcome the critical issues previously mentioned.

For this purpose in this work were evaluated several tools for sample treatment and data normalization in metabolomic analysis of urine samples using direct infusion electrospray mass spectrometry (DI-ESI-MS). It was studied several methods for urine treatment in order to reduce ion suppression, using solid phase extraction (SPE) with different sorbents, liquid-liquid extraction (LLE) and sample dilution. Moreover, ionic conductivity measurements and statistical preprocessing were compared for data normalization. In order to compare the effectiveness of these approaches for groups classification based on DIMS fingerprinting results, urine samples were studied from volunteers both men and women collected at different times of the day (fasting morning and after lunch). Finally, the most suitable approach was tested in a pilot study with urine samples from transgenic mice models of Alzheimer's disease (APP/PS1) in order to illustrate the potential of the methodology.

Materials and methods

Reagents and materials

All solvents used were HPLC-grade. Methanol and chloroform were purchased from Aldrich (Steinheim, Germany); acetic acid, ammonium acetate and dichloromethane were supplied by Merck (Darmstadt, Germany); and ammonia solution (32%) from VWR (Leuven, Belgium). Water was purified with a Milli-Q Gradient system (Millipore, Watford, UK). The SPE cartridges used were 3 ml columns, filled with 500 mg of DSC-18 (Supelco) for reverse phase extraction (C18), Bond Elut SI (Varian) for normal phase extraction (silica), and Isolute Multimode (IST) combining either non-polar (C18), strong cation exchange (SO_3^-) and strong anion exchange (NR_3^+) solid phases.

Sample collection

Two different sets of samples were used in this study. Human urine samples, collected from 11 volunteers (5 male and 6 female) fasting and after lunch, were used for evaluating the suitability of the different treatment and normalization procedures tested in this study. The second set was urine samples from transgenic mice models of Alzheimer's disease (APP/PS1) and wild-type controls, employed to validate the optimized methodology in a pilot study for the search of potential biomarkers of this disorder. APP/PS1 double transgenic mice (C57BL/6

background) were generated by crossing homozygotic PS1M146L transgenic mice with heterozygotic Thy1-APP751SL mice (Swedish: K670N, M671L and London: V717I FAD mutations) (Charles River, France), as previously reported [31]. On the other hand, age-matched wild-type mice of the same genetic background (C57BL/6) were purchased from Charles River Laboratory for their use as controls (WT). Male animals at 6 months of age were used for experiments (10 specimens per group). All samples were frozen immediately to -80ºC and stored until analysis.

Sample treatment

Three different strategies were tested for simultaneous desalting and extraction of metabolites from urine samples. In all cases, urine was thawed at room temperature and immediately centrifuged to remove particulate matter (6000 rpm, 10 minutes, 4 ºC). Before extraction, pH of these samples was adjusted to 7.

Sample dilution (SD). Urine was tenfold diluted with a 50 % (v/v) methanol/water mixture.

Liquid-liquid extraction (LLE). 500 µL of urine were mixed with the same volume of organic solvent (chloroform or dichloromethane), followed by vigorous vortexing for 5 min and centrifugation at 6000 rpm for 3 min at room temperature in order to collect the clean organic phase for analysis. Alternatively, 750 µL of a mixture of urine, methanol and organic solvent (1:1:1 v/v/v) was vigorously shaken by vortexing for 5 min and centrifuged at 6000 rpm for 3 min at room temperature.

Solid-phase extraction (SPE). Three SPE procedures were compared: reverse phase extraction (RP-SPE), normal phase extraction (NP-SPE) and mixed mode extraction (MM-SPE), this latter combining reverse phase sorbent with cationic and anionic exchangers. This extraction method requires sorbent wetting and equilibration before sample loading, followed by a washing step before the final elution of analytes. For this purpose, eluents were pumped through the column with a vacuum manifold (Visiprep^{TM}, Supelco). The solvents used for each SPE procedure are shown in Table 1.

Instrumentation

MS analyses were performed in a QSTAR XL Hybrid system (Applied Biosystems, Foster City, CA, USA) using an electrospray (ESI) source. The extracts were introduced into the mass spectrometer at 5 µL min^{-1} flow rates using an integrated apparatus pump and a 1000 µL volume Hamilton syringe. Data were obtained in positive ion mode, acquiring full scan spectra for 0.2 minutes in the m/z range 50-500 with 1.005 seconds of scan time. The ion spray voltage (IS) was set at 3300 V and nitrogen was used as curtain gas and nebulizer gas at flow rates about 1.13 L min^{-1} and 1.56 L min^{-1}, respectively. The source temperature was fixed at 60 ºC, with a declustering potential (DP) of 60 V and a focusing potential (FP) of 250 V. An AKTA-Prime system

(Amersham), integrated by a pump and conductivity detector, was used in flow injection mode for measurements of electrical conductivity. Extracts were injected in a 100 µl sample loop and were delivered to the detector by a carrier flow of water.

Data analysis

Markerview™ software (Applied Biosystems) was employed for peak picking and matching peaks across samples, in order to carry out the reduction of spectral data into a two-dimensional matrix of spectral peaks and peak intensities. For this, the peak search was done with a mass tolerance of 0.1 Da, and a minimum response of 10 counts was considered for filtering. In addition, missing values were substituted with the mean of the non-missing values across all samples for that peak. Then, data was processed in SIMCA-P™ software (version 11.5, published by UMetrics AB, Umeå, Sweden). Partial least squares discriminant analysis (PLS-DA) was performed to check the potential of the metabolomic approaches applied to urine samples for classification of samples in the groups of study. In addition, quality of the models was assessed by the R^2 and Q^2 values, supplied by the software, which provide information about the class separation and predictive power of the model, respectively. These parameters are ranged between 0 and 1, and they indicate the variance explained by the model for all the data analyzed (R^2) and this variance in a test set by cross-validation (Q^2). Statistical data normalization was carried out in the R software (freeware, http://www.r-project.org), using the locally weighted scatter plot smoothing (LOESS) normalization method, which adjusts the local median of log fold changes of peak intensities between samples in a data set to be approximately zero across the whole peak intensity range [32]. In addition, the heteroscedasticity of noise in peak intensities, characterized by increasing technical variance as a function of increased signal intensity, was corrected by means of a logarithmic-based variance-stabilization transformation, to make peak intensity consistent across the whole intensity range [32,33].

Results and discussion

The comparison of metabolomic profiles obtained with the different tested procedures demonstrated the importance of a suitable choice of the extraction protocol for obtaining comprehensive results when DIMS is used as metabolomic approach. In addition, a considerable inter-sample variability was observed, showing the need of using normalization strategies. In order to determine the most suitable approach for urine metabolomics, statistical analysis (PLS-DA) of data from the first sample set (men and women, and different times of collection - morning and midday) was performed for sample classification according to the groups of study.

Then, the applicability of the optimized methodology was checked in a pilot study with APP/PS1 transgenic mice and wild-type controls.

Classification of human urine samples using non-normalized results

Application of treatment procedures previously described to urine samples provided different DIMS metabolomic profiles, showing that extraction of metabolites is strongly dependent on the selectivity of technique, which may result in biased fingerprinting. Thus, data obtained by simple dilution of urine samples with 50% water/methanol mixture could be considered as reference fingerprint of this type of sample, since no losses of metabolites occurs with this treatment, which allows obtaining comprehensive spectral profiles (Fig. 1a). The optimal dilution factor was estimated considering the number of peaks and total intensity of signal in overall mass spectrum resulting after successive dilutions. In this way, only a dilution higher than tenfold provoked a significant decrease in sensitivity of metabolomic profiles, so this value was selected for further experiences. When urine was liquid-liquid extracted with chloroform or dichloromethane very similar metabolic profiles were obtained, but the number and intensities of signals increase when the extraction was performed adding methanol (Fig. 1b, for chloroform-methanol), since methanol acts as "phase transfer agent" promoting the extraction of more polar analytes from aqueous to organic phase. However, comparing these results with those from urine dilution (Fig. 1a), the number of extracted metabolites considerably decreased due to the loss of polar compounds in these non-aqueous solvents, such as chloroform or dichloromethane. Finally, when urine is solid-phase extracted, different profiles are obtained depending on the use of normal or reverse extraction phases, since the extraction is somewhat selective towards polar or lipophilic metabolites respectively, as is shown in Fig. 1c and 1d. On the other hand, when the mixed mode sorbent is used, the mass spectra of the extract exhibits a higher number of signals (Fig. 1e), similarly to that obtained from diluted samples, demonstrating the capability of the mixed mode sorbent for the global recovery of urinary metabolites.

In addition to the visual inspection of metabolomic profiles obtained for each pretreatment procedure, multivariate statistics allowed evaluating the suitability of the different urine extraction protocols for metabolomic fingerprinting. In this way, MS profiles were submitted to partial least squares discriminant analysis, and 3D scores plots of the three first components (in five components models) were represented in order to visualize trending and grouping of samples (Fig. 2, for the best procedures in terms of metabolite coverage observed in MS profiles). In this way, the best discrimination between the four groups was obtained with sample dilution and mixed mode SPE extraction (Fig. 2a and 2c). Moreover, the higher statistical quality of these models can be corroborated by the parameters of separation and predictive power (shown in Figure 4,

under the respective scores plots), which also support the advantage of SD and MM-SPE over other procedures. Furthermore, in order to evaluate the chemical coverage of the metabolome obtained with these procedures compared by multivariate statistics (SD, LLE, MM-SPE), a number of representative metabolites from metabolomic fingerprints were identified (Table 2). As can be observed, all these metabolites were detected using the three extraction procedures, but higher sensitivity was obtained with diluted samples and, to a lesser extent, solid phase extraction (in terms of peak intensity), corroborating the higher potential of these methods for high-throughput metabolomic analysis.

Classification of human urine samples after ionic conductivity normalization

The ionic conductivity of urine extracts is a parameter of great interest, since provides information about effectiveness of salts removing from the sample after treatment, and allows data normalization in relation to physiological dilution rate of urine. Thus, conductivity was measured in diluted samples and extracts from SPE, resulting in high dispersion of values for urine dilution samples (SD), that range from 0.08 to 0.56 mS cm^{-1} with RSD of 39 %, which denotes a great physiological variability between samples, and low conductivity in reverse and normal phase extracts (RP-SPE and NP-SPE), in which the mean values of conductivity are 0.20 and 0.08 mS cm^{-1}, although in this case the conductivity is difficult to normalize due to the wide variability of results (RSD of 48 and 63% for RP-SPE and NP-SPE, respectively). In mixed mode extraction (MM-SPE), the conductivity, with mean values of 0.49 mS cm^{-1}, is difficult to reduce probably due to the use of buffer solutions in the elution step, however, the reproducibility is better (RSD=18%).

Therefore, it can be concluded that urine desalting is more effective by using reverse and normal phase extraction, but the reproducibility is considerably higher with mixed mode sorbents. Then, these values of ionic conductivity were employed for the normalization of signal intensities in mass spectra to improve the discrimination between the groups of study. However, for diluted samples and the extracts from RP-SPE and NP-SPE treatments, the separation between groups was reduced comparing with non-normalized data, while for MM-SPE the statistical model obtained after normalization was analogous to that non-normalized, with R^2=0.979 and Q^2=0.455 (Fig. 3a and 3b).

Classification of human urine samples after statistical normalization

Finally, statistical normalization of data by LOESS method was used in order to obtain optimal scaling factors for each sample based on the complete dataset, assuming that metabolomic profiles should be similar between different samples in absence of differences in the excreted volume of urine. This approach provided slightly

better discrimination models compared to non-normalized ones, with higher R^2 and Q^2 values and scores plots with better separation between groups (Fig. 3c, 3d and 3e). Furthermore, discrimination was clearer in models built with data from MM-SPE (R^2=0.988, Q^2=0.517) and diluted samples (SD) (R^2=0.983, Q^2=0.559), similarly to that found with non-standardized data.

In summary, it could be concluded that sample dilution is the most suitable procedure for urine treatment before analysis by direct infusion mass spectrometry, in terms of simplicity, sensitivity and metabolomic coverage (Table 2). Furthermore, statistical normalization by LOESS showed the higher potential for correcting inter-sample variability, facilitating group discrimination in PLS-DA models. Thereby, it can be observed that the number of discriminant metabolites (VIP>2.0) increases from 18 to 25 when VIP ranking plots from non-normalized data and results statistically normalized are compared (Fig. 4), demonstrating the usefulness of LOESS normalization to extract the relevant biological information from this data set.

Application of DIMS metabolomics to a pilot study with Alzheimer's disease transgenic mice

Direct analysis of diluted samples followed by statistical normalization of data (LOESS) has been demonstrated to be the most reliable and simple approach for high throughput fingerprinting of urine by direct infusion electrospray mass spectrometry, considering results shown in previous sections. In order to evaluate the potential of this methodology in a real metabolomic investigation, urine samples from transgenic mice of Alzheimer's disease (APP/PS1) and wild-type controls were analyzed in the same way. Alzheimer's disease (AD) is the most common neurodegenerative disorder worldwide, whose exact etiology and pathogenesis are not clear. For a better understanding of pathogenic mechanisms occurring, transgenic mouse models have been developed, among which the double mutant transgenic line APP/PS1 has gained great importance [34]. The potential of metabolomics for the discovery of biomarkers in AD has been previously reported [35], but the use of urine has been only scarcely considered [36]. Thus, the application of the methodology optimized in the present work could represent a new tool in the search of potential biomarkers using a very simple and easily available sample in clinical laboratory as is urine. In this pilot study, ten specimens were included per group (transgenic mice - TG; and wild-type controls - WT). Partial least squares discriminant analysis provided a good classification of samples in the two expected groups (Fig. 5a), with very high statistic parameters of class separation and predictive power (R^2=0.999 and Q^2=0.99). In addition, numerous signals varied significantly between groups, as shown in the Variable Importance plot (Fig. 5b). Therefore, these preliminary results demonstrate the potential of high throughput direct infusion metabolomics in urine samples for the study of complex diseases such as AD, and open the door to future studies for biomarkers discovery based in this fluid.

Conclusions

The importance of sample treatment and data normalization in the analysis of urine by direct infusion mass spectrometry for metabolomic studies has been evaluated. Ion suppression must be reduced in order to obtain valuable results, for which different protocols of sample preparation were studied. Simple tenfold dilution of samples and solid phase extraction in a mixed-mode sorbent (MM-SPE) provided the best results for comprehensive metabolomic fingerprinting and grouping of samples in the subsequent statistical analysis. On the other hand, liquid-liquid extraction with organic solvents and solid phase extraction with reverse and normal phases, which involves a more selective recovery of metabolites, provided more biased metabolomic profiles. Moreover, the suitability of a normalization step was evaluated in order to correct the physiological variability of urine samples. Statistical processing of non-normalized data allowed discrimination between groups in diluted urine and MM-SPE extracts. Normalization based on conductivity provided good statistical models only for MM-SPE extracts, in which conductivity measures showed that this is the only procedure with reproducible desalting power. On the other hand, statistical normalization by LOESS showed the higher potential for these purposes, improving significantly the quality of the metabolomic data. However, a limitation of this study is the absence of a protocol for diet standardization prior to sample collection, as proposed by Favé et al. in order to reduce inter-subject variability [37]. Furthermore, 24-h urine samples from the same subjects were not available in our study design in order to compare with samples randomly collected, although these latter are usually more employed in metabolomic studies due to easier sampling. Finally, the optimized approach based on direct analysis of diluted samples followed by statistical normalization of data was applied to a pilot study with samples from APP/PS1 transgenic mice, demonstrating the potential of this methodology for high throughput metabolomics of urine samples.

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References

[1] O. Fiehn, Metabolomics–the link between genotypes and phenotypes, Plant Mol. Biol. 48 (2002) 155-171.

[2] D.S. Wishart, Metabolomics: applications to food science and nutrition research, Trends Food Sci. Tech. 19 (2008) 482-493.

[3] P. Goldsmith, H. Fenton, G. Morris-Stiff, N. Ahmad, J. Fisher, K.R. Prasad, Metabonomics: A useful tool for the future surgeon, J. Surg. Res. 160 (2010) 122-132.

[4] K. Dettmer, P.A. Aronov, B.D. Hammock, Mass spectrometry-based metabolomics, Mass Spectrom. Rev. 26 (2007) 51-78.

[5] H.J. Issaq, E. Abbott, T.D. Veenstra, Utility of separation science in metabolomic studies, J. Sep. Sci. 31 (2008) 1936-1947.

[6] W.B. Dunn, Current trends and future requirements for the mass spectrometric investigation of microbial, mammalian and plant metabolomes, Phys. Biol. 5 (2008) 011001.

[7] L. Lin, Q. Yu, X. Yan, W. Hang, J. Zheng, J. Xing, B. Huang, Direct infusion mass spectrometry or liquid chromatography mass spectrometry for human metabonomics? A serum metabonomic study of kidney cancer, Analyst 135 (2010) 2970-2978.

[8] P.G. Lokhov, M.I. Dashtiev, S.A. Moshkovskii, A.I. Archakov, Metabolite profiling of blood plasma of patients with prostate cancer, Metabolomics 6 (2010) 156-163.

[9] P.G. Lokhov, O.N. Kharybin, A.I. Archakov, Diagnosis of lung cancer based on direct-infusion electrospray mass spectrometry of blood plasma metabolites, Int. J. Mass Spectrom. 309 (2012) 200-205.

[10] R. Gonzalez-Dominguez, T. Garcia-Barrera, J.L. Gomez-Ariza, Metabolomic approach to Alzheimer's disease diagnosis based on mass spectrometry, Chem. Papers 66 (2012) 829–835.

[11] R. Gonzalez-Dominguez, T. Garcia-Barrera, J.L. Gomez-Ariza, Combination of metabolomic and phospholipid-profiling approaches for the study of Alzheimer's disease, J. Proteomics (2014) *in press.*

[12] J.A. Simerville, W.C Maxted, J.J. Pahira, Urinalysis: A comprehensive review, Am. Fam. Physician 71 (2005) 1153-1162.

[13] M.A. Fernández-Peralbo, M.D. Luque de Castro, Preparation of urine samples prior to targeted or untargeted metabolomics mass-spectrometry analysis, TrAC-Trends Anal. Chem. 41 (2012) 75-85.

[14] T.M. Annesley, Ion suppression in mass spectrometry, Clin. Chem. 49 (2003) 1041-1044.

[15] S.S. Que-Hee, Biological monitoring: An introduction, John Wiley & Sons, New York, 1993.

[16] R. Bonfiglio, R.C. King, T.V. Olah, K. Merkle, The effects of sample preparation methods on the variability of the electrospray ionization response for model drug compounds, Rapid Commun. Mass Spectrom. 13 (1999) 1175-1185.

[17] V. Walker, G.A. Mills, Solid-phase extraction in clinical biochemistry, Annals Clin. Biochem. 39 (2002) 464-477.

[18] F. Michopoulos, L. Lai, H. Gika, G. Theodoridis, I. Wilson, UPLC–MS-based analysis of human plasma for metabonomics using solvent precipitation or solid phase extraction, J. Proteome Res. 8 (2009) 2114-2121.

[19] H. Idborg-Bjorkman, P. Edlund, O.M. Kvalheim, I. Schuppe-Koistinen, S.P. Jacobsson, Screening of biomarkers in rat urine using LC/electrospray ionization-MS and two-way data analysis, Anal. Chem. 75 (2003) 4784-4792.

[20] A. Namera, S. Yamamoto, T. Saito, S. Miyazaki, H. Oikawa, A. Nakamoto, M. Nagao, Simultaneous extraction of acidic and basic drugs from urine using mixed-mode monolithic silica spin column bonded with octadecyl and cation-exchange group, J. Sep. Sci. 34 (2011) 2232-2239.

[21] B.M. Warrack, S. Hnatyshyna, K.H. Otta, M.D. Reilya, M. Sanders, H. Zhang, D.M. Drexler, Normalization strategies for metabonomic analysis of urine samples, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 877 (2009) 547-552.

[22] V. Chadha, U. Garg, U.S. Alon, Measurement of urinary concentration: a critical appraisal of methodologies, Pediatr. Nephrol. 16 (2001) 374-382.

[23] E.J. Saude, D. Adamko, B.H. Rowe, T. Marrie, B.D. Sykes, Variation of metabolites in normal human urine, Metabolomics 3 (2007) 439-451.

[24] D. Ryan, K. Robards, P.D. Prenzler, M. Kendall, Recent and potential developments in the analysis of urine: A review, Anal. Chim. Acta. 684 (2011) 17-29.

[25] F. Dieterle, A. Ross, G. Schlotterbeck, H. Senn, Probabilistic quotient normalization as robust method to account for dilution of complex biological mixtures. Aplication in ^{1}H-NMR metabolomics, Anal. Chem. 78 (2006) 4281-4290.

[26] S.M. Kohl, M.S. Klein, J. Hochrein, P.J. Oefner, R. Spang, W. Gronwald, State-of-the art data normalization methods improve NMR-based metabolomic analysis, Metabolomics 8 (2012) 146-160.

[27] M. Beckmann, D.P. Enot, D.P. Overy, I.M. Scott, P.G. Jones, D. Allaway, J. Draper, Metabolite fingerprinting of urine suggests breed-specific dietary metabolism differences in domestic dogs, Br. J. Nutr. 103 (2010) 1127-1138.

[28] A.J. Lloyd, G. Favé, M. Beckmann, W. Lin, K. Tailliart, L. Xie, J.C. Mathers, J. Draper, Use of mass spectrometry fingerprinting to identify urinary metabolites after consumption of specific foods, Am. J. Clin. Nutr. 94 (2011) 981-991.

[29] G. Favé, M. Beckmann, A.J. Lloyd, S. Zhou, G. Harold, W. Lin, K. Tailliart, L. Xie, J. Draper, J.C. Mathers, Development and validation of a standardized protocol to monitor human dietary exposure by metabolite fingerprinting of urine samples, Metabolomics 7 (2011) 469-484.

[30] M. Hasegawa, S. Takenaka, M. Kuwamura, J. Yamate, S. Tsuyama, Urinary metabolic fingerprinting for amiodarone-induced phospholipidosis in rats using FT-ICR MS, Exp. Toxicol. Pathol. 59 (2007) 115-120.

[31] V. Blanchard, S. Moussaoui, C. Czech, N. Touchet, B. Bonici, M. Planche, T. Canton, I. Jedidi, M. Gohin, O. Wirths, T.A. Bayer, D. Langui, C. Duyckaerts, G. Tremp, L. Pradier, Time sequence of maturation of dystrophic neuritis associated with Abeta deposits in APP/PS1 transgenic mice, Exp. Neurol. 184 (2003) 247-263.

[32] K.A. Veselkov, L.K. Vingara, P. Masson, S.L. Robinette, E. Want, J.V. Li, R.H. Barton, C. Boursier-Neyret, B. Walther, T.M. Ebbels, I. Pelczer, E. Holmes, J.C. Lindon, J.K. Nicholson, Optimized preprocessing of ultra-performance liquid chromatography/mass spectrometry urinary metabolic profiles for improved information recovery, Anal. Chem. 83 (2011) 5864-5872.

[33] M. Sysi-Aho, M. Katajamaa, L. Yetukuri, M. Orešič, Normalization method for metabolomics data using optimal selection of multiple internal standards, BMC Bioinformatics 8 (2007) 93-110.

[34] A.M. Hall, E.D. Roberson, Mouse models of Alzheimer's disease, Brain Res. Bull. 88 (2012) 3-12.

[35] C. Ibáñez, C. Simó, A. Cifuentes, Metabolomics in Alzheimer's disease research, Electrophoresis 34 (2013) 2799-2811.

[36] K. Fukuhara, A. Ohno, Y. Ota, Y. Senoo, K. Maekawa, H. Okuda, M. Kurihara, A. Okuno, S. Niida, Y. Saito, O. Takikawa, NMR-based metabolomics of urine in a mouse model of Alzheimer's disease: identification of oxidative stress biomarkers, J. Clin. Biochem. Nutr. 52 (2013) 133-138.

[37] G. Favé, M. Beckmann, A.J. Lloyd, S. Zhou, G. Harold, W. Lin, K. Tailliart, L. Xie, J. Draper, J.C. Mathers, Development and validation of a standardized protocol to monitor human dietary exposure by metabolite fingerprinting of urine samples, Metabolomics 7 (2011) 469-484.

Figure legends

Fig. 1 Metabolomic fingerprints from diluted urine (a), LLE extract using chloroform/methanol (b) and SPE extracts using NP-SPE (c), RP-SPE (d) and MM-SPE (e)

Fig. 2 3D scores plots and statistical parameters of non-normalized data for: diluted samples (a), LLE using chloroform/methanol (b), and MM-SPE (c)

Fig. 3 3D scores plots of normalized data based on ionic conductivity for: diluted samples (a), and MM-SPE (b); and statistically normalized for: diluted samples (c), LLE using chloroform/methanol (d), and MM-SPE (e)

Fig. 4 Variable Importance plots for non-normalized data (a) and data normalized with LOESS (b)

Fig. 5 Partial least squares discriminant analysis of data from the pilot study with Alzheimer's disease transgenic mice: scores plot (a), and Variable Importance plot (b)

Table 1.- Experimental condition for SPE procedures

	RP-SPE	**NP-SPE**	**MM-SPE**
Conditioning	2ml methanol	2ml methanol	2ml methanol
Equilibration	2ml water	2ml methanol 5%	-
Sample loading	1ml urine	1ml urine	1.5ml urine
Washing	2ml water	2ml methanol 95%	2ml water
Elution	1ml methanol	1ml methanol 5%	1. 0.5ml methanol 2. 0.5ml ammonium acetate 10 mM (pH 3) 3. 0.5ml ammonia 5% (in methanol)

Table 2 Peak intensities of representative metabolites identified in urine samples detected by sample dilution (SD), liquid-liquid extraction (LLE) and mixed mode solid-phase extraction (MM-SPE)

metabolite	m/z (Da)	peak intensity		
		SD	LLE	MM-SPE
trimethylamine	60.079	278.7	1.5	54.4
urea	61.037	13361.6	60.8	380.4
trimethylamine N-oxide	76.074	5813.9	92.8	686.1
alanine	90.055	121.6	0.3	7.0
glycerol	93.051	26.0	0.6	11.6
choline	104.105	187.7	3.3	53.9
serine	106.054	39.6	11.8	49.0
creatinine	114.064	18741.6	2137.2	15650.5
proline	116.075	133.5	14.5	77.5
valine	118.082	224.7	5.8	65.5
threonine	120.069	29.9	4.2	36.4
cysteine	122.031	55.9	5.3	30.9
taurine	126.025	128.5	16.0	55.7
pipecolic acid	130.098	229.9	8.7	71.1
creatine	132.074	440.0	7.2	72.4
aspartic acid	134.051	170.3	2.5	24.9
trigonelline	138.052	1804.4	8.9	233.6
histidine	156.074	1262.1	6.0	87.8
carnitine	162.108	1458.9	2.9	152.4
phenylalanine	166.082	5391.8	20.4	472.2
methylhistidine	170.086	1840.4	8.1	113.9
arginine	175.116	426.8	2.7	42.4
N-acetyl-aspartic acid	176.055	272.1	4.3	20.2

glucose	181.079	527.4	38.0	811.2
tyrosine	182.076	1985.7	11.4	330.8
phosphocholine	184.081	378.2	43.6	133.9
acetylcarnitine	204.118	532.1	4.2	99.7
carnosine	227.122	1183.3	86.5	459.1
uridine	245.081	316.3	4.1	44.1
uridine monophosphate	325.049	492.4	0.5	196.2

Figure 1

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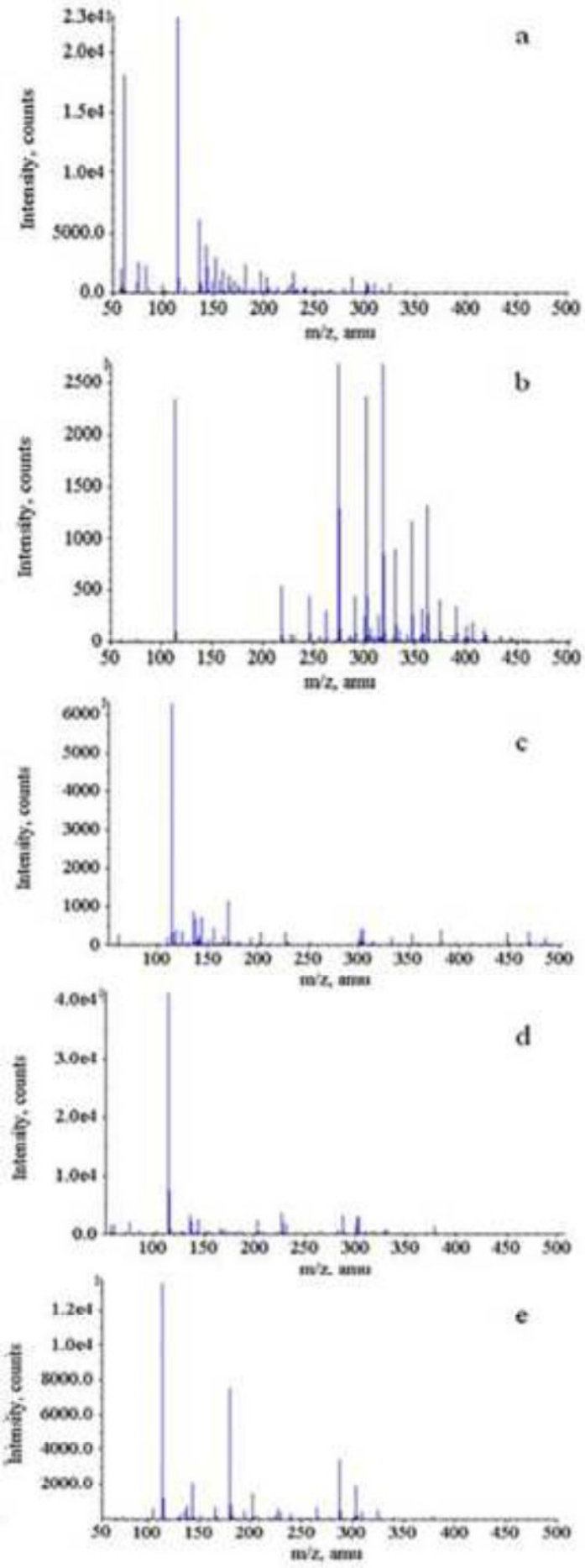

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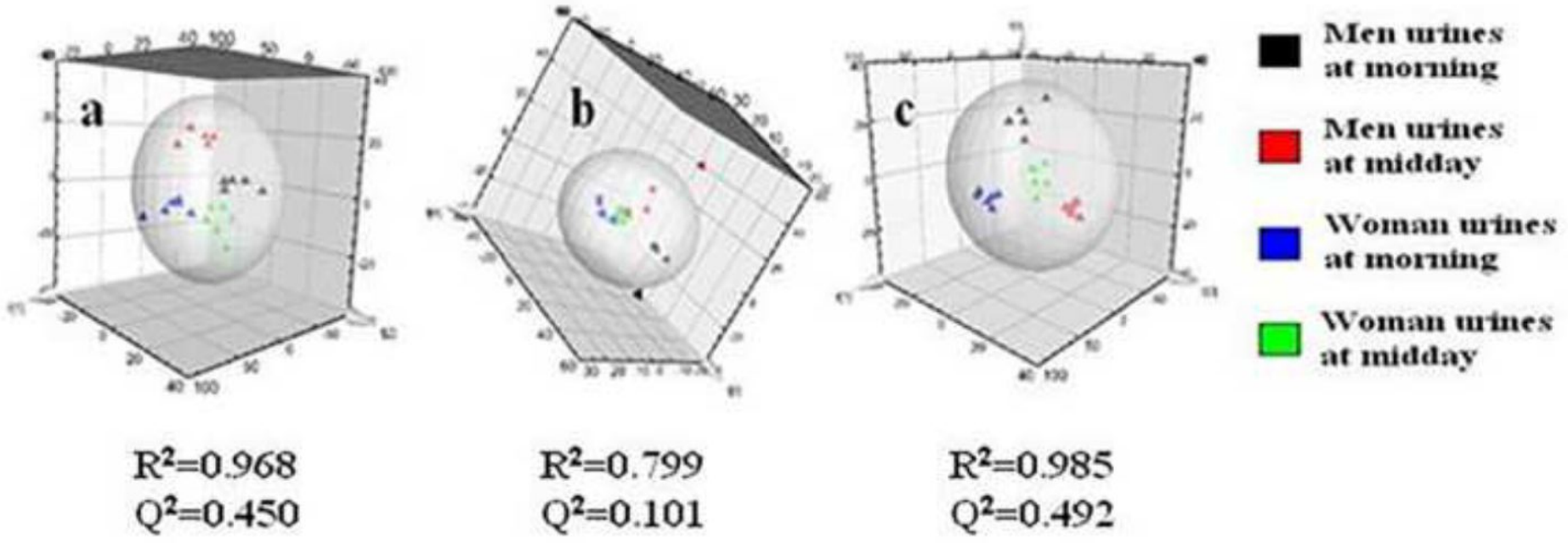

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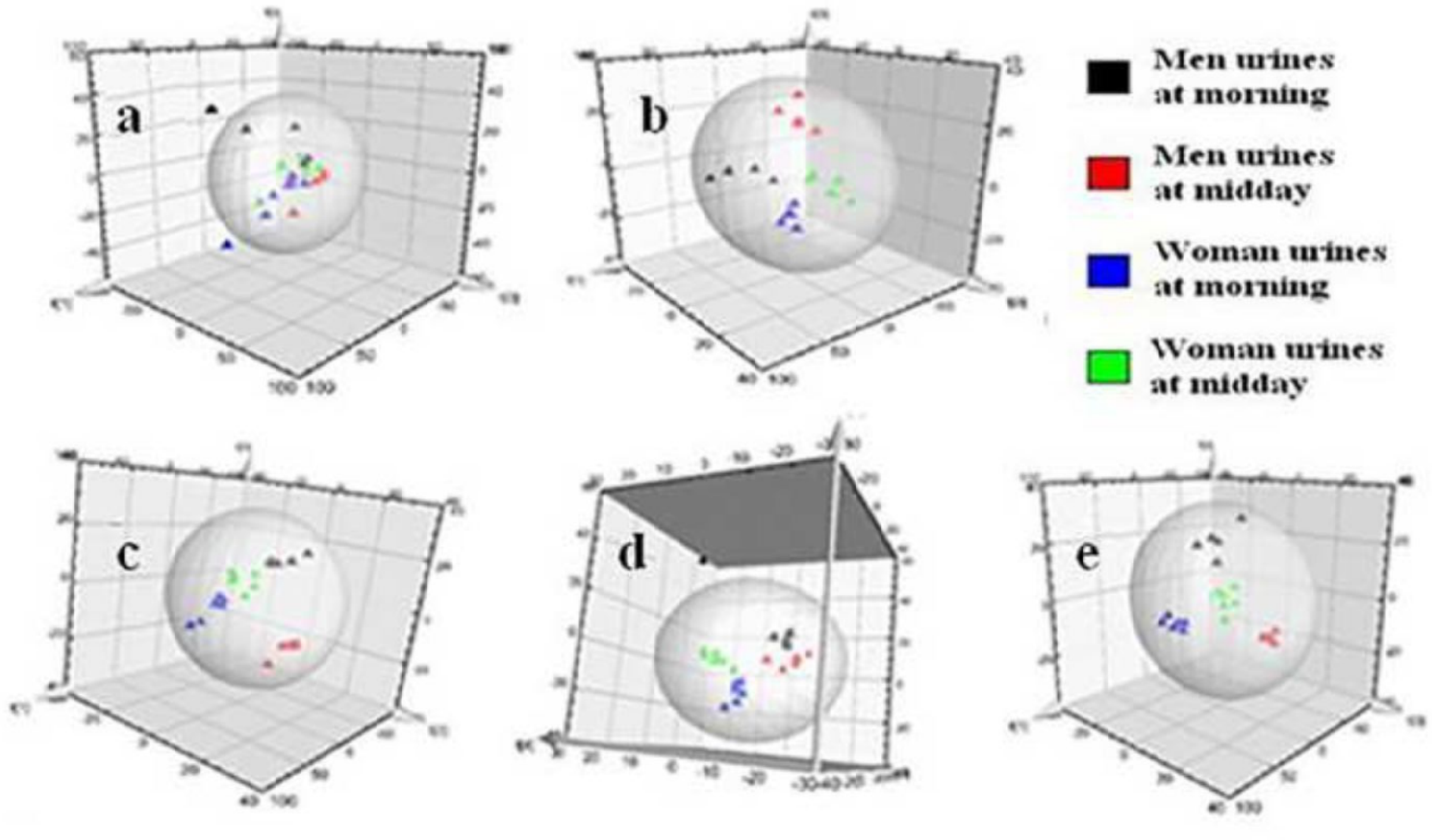

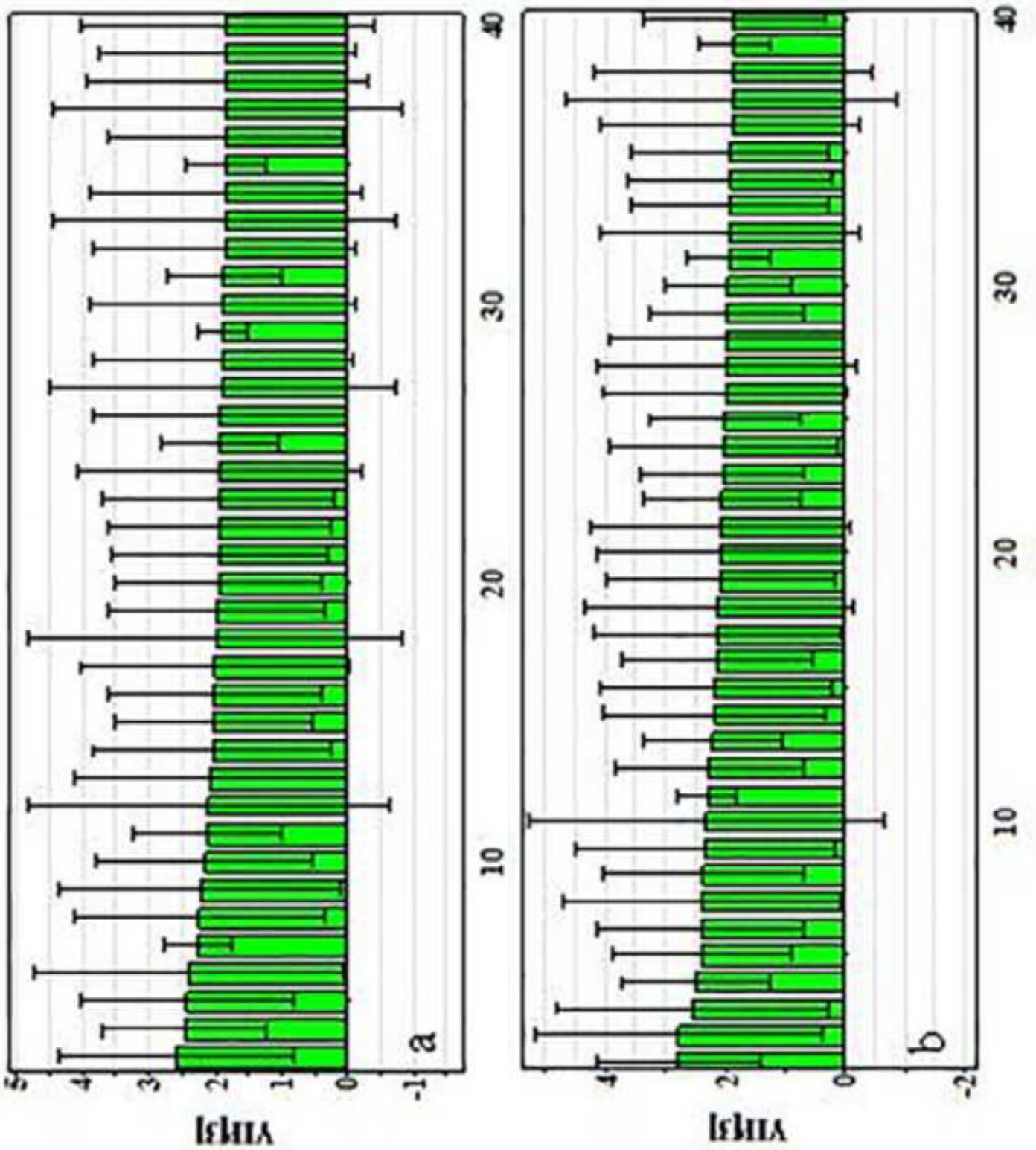

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Figure 5

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